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# EVIDENCE FOR GLUCOCORTICOID RECEPTORS IN CULTURED RABBIT ARTICULAR CHONDROCYTES

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## 1. Introduction

It is now widely accepted that glycocorticoids initiate their action by binding to specific receptor proteins in the cytoplasm of target tissues [1–4]. Locally injected glucocorticoids can improve osteoarthrosis or acute refractory rheumatoid arthritis, but they may also induce progressive joint destruction. Their effects on joints could be mediated through direct hormonal influence on chondrocytes, the constitutive cell of cartilage. Until now glucocorticoid receptors have been only described on embryonic chick growth cartilage [5], but they have been not shown yet in cultured chondrocytes. The purpose of this study was to determine the presence of glucocorticoid receptors in cultured chondrocytes obtained from rabbit articulations and to describe the main characteristics of [$^3$H]dexamethasone binding in a whole cell assay.

## 2. Materials and methods

### 2.1. *Cell culture*

Cartilage was taken from the shoulder and the knee joints of rabbits (Fauve de Bourgogne), aged from 1–2 months. Chondrocytes were enzymatically released from cartilage slices using the technique in [6]. Isolated cells were then cultured in Ham F 12 medium (Gibco) supplemented with 10% fetal calf serum, 10 IU penicillin/ml, 10 $\mu$g streptomycin/ml and maintained at 37°C in an atmosphere of 95% air, 5% $CO_2$. When monolayers reached confluence, the primary culture was trypsinized. Cells were then seeded in 75 cm$^2$ tissue culture flasks at 1.5 × 10$^6$ cells/flask with 15 ml complete medium. In all experiments the cells were treated on day 6 of culture including the last 12 h in a medium without serum.

### 2.2. *[$^3$H]Dexamethasone binding*

At the time of the experiment, the medium was replaced by new medium without serum containing various concentrations of [$^3$H]dexamethasone (25 Ci/mmol, Amersham). To determine non-specific binding, parallel incubations were carried out with 1000-fold molar excess of non-radioactive dexamethasone (Sigma). After 40 min at 37°C, the medium from each flask was collected and an aliquot used to determine free dexamethasone. The flasks were then placed on ice, and the monolayer washed twice with PBS at 0°C, two washes with PBS for 15 min at 0°C were used to allow unspecifically bound radioactivity to diffuse out of the intact cells. The last pellet was resuspended in 0.5 ml distilled water and maintained at 0°C for 15 min until cell disruption. Aliquots of 200 $\mu$l were used to determine DNA content by the method in [7] and the remaining suspension counted in a liquid scintillation counter to determine cellular bound radioactivity.

### 2.3. *Competitive binding studies*

Competition for binding with whole cells was carried out as for binding of [$^3$H]dexamethasone alone except that the incubation media contained both [$^3$H]dexamethasone at a single concentration ($10^{-8}$ M) and the unlabelled competing steroid at various concentrations ($10^{-8}$–$10^{-4}$ M). The residual binding in the presence of competitor was expressed as % of binding of [$^3$H]dexamethasone at $10^{-8}$ M alone.

Unlabelled steroids (Sigma) were dissolved in

ethanol–benzene (8:2) at $5 \times 10^{-3}$ M. Levels of $10^{-4}$–$10^{-8}$ M were obtained after drying an aliquot under nitrogen gas then dissolving the residue in ethanol. The final concentration was adjusted with culture medium. Incubation medium of these steroids contained <1% ethanol which did not affect dexamethasone binding. In these conditions 17 β-estradiol was insoluble at $10^{-4}$ M and therefore was not tested.

## 3. Results

### 3.1. *Time course of [$^3$H]dexamethasone binding*

Preliminary experiments were done to determine the saturation time of binding at 37°C with a single high concentration of [$^3$H]dexamethasone ($10^{-8}$ M). The time course of binding for the 2 types of sites observed in the whole cell assay are reported in fig.1. Saturation of unspecific binding sites rapidly occurred after 5 min incubation. In the case of both total and specific binding, the bound radioactivity increased rapidly over 15 min reaching a plateau after 20 min incubation at 37°C.

### 3.2. *Saturation curve*

Equilibrium binding at increasing concentrations of [$^3$H]dexamethasone ($10^{-9}$–$5 \times 10^{-8}$ M) was done in intact cells attached to the culture flask, incubated for 40 min at 37°C. At the end of the incubation period, separation of bound from free steroid was

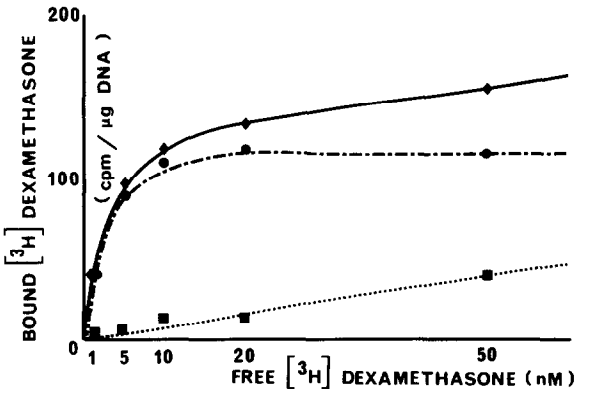


Fig.2. [$^3$H]Dexamethasone binding of cultured chondrocytes. Intact cells in monolayer were incubated for 40 min at 37°C with increasing concentrations of [$^3$H]dexamethasone (1–50 nM). Parallel incubations were done in the presence of 1000-fold molar excess of non-radioactive dexamethasone in order to determine non-specific binding. Specific binding (●– - – - –●) represented the difference between total binding (♦——♦) and non-specific binding (■- - -■). Each value expressed as cpm bound/µg DNA is the mean of 3 expt.

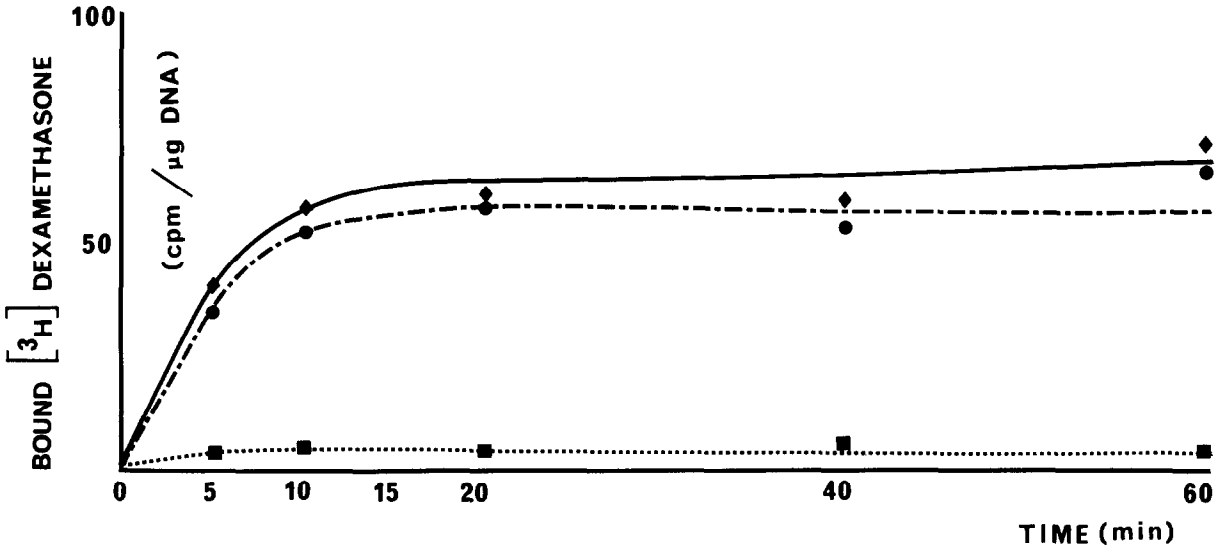


Fig.1. Time course of [$^3$H]dexamethasone binding at 37°C. Medium containing [$^3$H]dexamethasone ($10^{-8}$ M) was added to each culture flask and cells incubated at 37°C for various times (5–60 min). Parallel incubations were done with medium containing [$^3$H]dexamethasone ($10^{-8}$ M) plus unlabelled dexamethasone ($10^{-5}$ M) to determine non-specific binding. At each time the specific binding (●– - – - –●) represents the difference between the total binding (♦——♦) and the non-specific binding (■- - -■). Each point represents a mean of 3 individual expt.

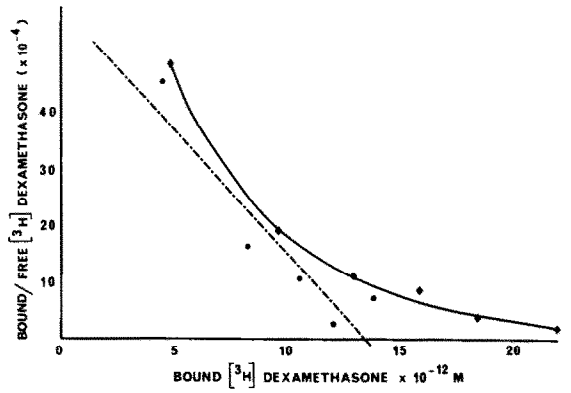


Fig.3. Scatchard analysis of [$^3$H]dexamethasone binding in cultured chondrocytes. The results of a typical experiment are shown as Scatchard plots analyzed into total binding (♦——♦) and specific binding (●- - - - -●). The no. cells was $2.85 \times 10^6$ in 15 ml medium. The app $K_d$ is $2.3 \times 10^{-9}$ M and the concentration of specific sites calculated from the intercept of the straight line with the abscissa is 43 260 sites/cell.

effected by 2 washes for 15 min at 0°C with cold saline solution as described in section 2.

Fig.2 shows that binding could be resolved into two components: a high affinity component, saturable at $2 \times 10^{-8}$ M that could be displaced by excess non-radioactive steroid, and a low affinity component which was not saturated up to $5 \times 10^{-8}$ M and was not displaced by an excess of non-radioactive steroid.

The binding data for each experiment were calculated from Scatchard plots [8] using linear regression analysis. Those values were consistent with the existence of a single population of high affinity binding sites (fig.3). The app. $K_d$ was $1.6–3.3 \times 10^{-9}$ M. Using an average DNA content of 10.8 $\mu$g/$10^6$ cells it could be calculated that ~40 000 binding sites were present/cell.

### 3.3. *Specificity of the dexamethasone binding sites*

The specificity of binding to intact cells was investigated by using various non-radioactive steroids at $10^{-8}–10^{-4}$ M to study displacement of [$^3$H]dexamethasone. The means of 2 or 3 series of experiments for each steroid studied are given in fig.4. The figures clearly show that dexamethasone competed very strongly: unlabelled dexamethasone ($10^{-8}$ M) displaced ~50% of the bound dexamethasone. At 10-fold concentration of unlabelled dexamethasone ($10^{-7}$ M) most of the binding was inhibited. 50% of binding was inhibited by corticosterone and progesterone at ~$10^{-6}$ M. Testosterone and 17 $\beta$-estradiol competed only poorly, with 50% inhibition at ~$10^{-5}$ M.

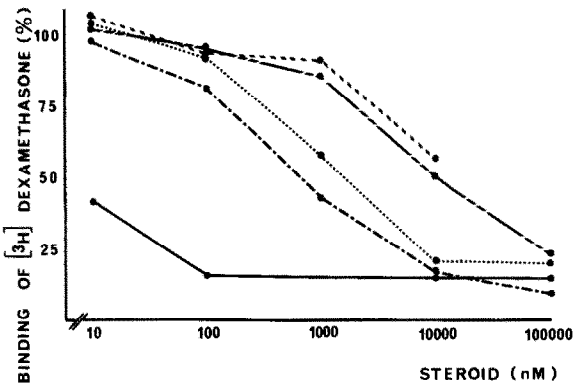


Fig.4. Competition by various steroids for binding to glucocorticoid receptors in cultured chondrocytes. The cells were incubated for 40 min at 37°C either in the presence of [$^3$H]dexamethasone alone (10 nM) or with [$^3$H]dexamethasone plus various concentrations of unlabelled steroids. The binding of [$^3$H]dexamethasone alone is plotted at 100% and the binding of [$^3$H]dexamethasone in the presence of competitors expressed as a % thereof: dexamethasone (●——●), corticosterone (●- - - - -●), progesterone (● · · · ●), testosterone (●— - - —●) and 17 $\beta$-estradiol (●- - ●).

## 4. Discussion

In these experiments, [$^3$H]dexamethasone binding was studied in a whole cell assay. This procedure has been used on various cell types: lymphoid cell lines [9]; reno-medullary interstitial cells [10]; thymocytes [11]; macrophages [12]. In [9,11] the results obtained by the whole cell assays were compared to those from cell free binding studies. They concluded that it was a reliable method to measure the affinity and the number of steroid binding sites. Furthermore, repeated washes of the intact cells to dissociate low affinity binding yielded a very low background count of the unspecifically bound radioactivity, usually <25% of the total binding of radioactive hormone. In these conditions, the method appeared to be sensitive, convenient and has the advantage of requiring ~$2–3 \times 10^6$ cells/flask.

Here, glucocorticoid binding was examined with dexamethasone as the ligand. This synthetic glucocorticoid has the advantage of not binding to transcortin. Experiments were also done with cells after 12 h in a medium without serum to decrease interference from exogenous steroid: thus accurate gluco-

corticoid concentrations could be determined. These data demonstrate that chondrocytes in culture and at the first passage, contained dexamethasone binding sites which possess most of the characteristics of glucocorticoid receptors.

These receptors have a high affinity for [$^3$H]dexamethasone with $K_d \sim 2.5 \times 10^{-9}$ M. In these conditions, a limited number of binding sites (40 000 sites/cell) which are saturated at low concentration ($2 \times 10^{-8}$) appeared. These results could be related to those obtained in vivo [5] with embryonic chick growth cartilage. A cytoplasmic component with $K_d$ $7.4 \times 10^{-9}$ M and saturation at $2.5 \times 10^{-8}$ M was described [5].

As determined by competition experiments, these receptors exhibit high specificity for dexamethasone and corticosterone, a moderate affinity for progesterone and virtually no affinity for sex steroids like testosterone and estradiol.

However corticosterone at 10 and 100 mM exhibited a lower affinity for the receptors than did dexamethasone. This phenomenon was also observed in whole cell assays for other cell types [11,13,14] and appeared different from the results using cell free systems. The discrepancy found with bone cell culture [13], might be the result of a reduced capacity or ability of corticosterone to enter the cytoplasm compartment of the intact cells rather than a true difference in receptor properties.

In conclusion, our study carried out on intact cells in vitro, demonstrates by a simple and convenient method, the presence of glucocorticoid receptors in cultured rabbit articular chondrocytes with the main characteristics of those described in other tissue cultures.

Such receptors could be responsible for the therapeutic improvement, whereas unwanted reactions sometimes observed after locally injected high doses of glucocorticoids, could be related to a toxic effect not mediated by these specific receptors.

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